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TITLE

Plasticity of tyrosine hydroxylase and serotonergic systems in the regenerating spinal cord in adult zebrafish.

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ABSTRACT

Monoaminergic innervation of the spinal cord has important modulatory functions for locomotion. Here we performed a quantitative study to determine the plastic changes of tyrosine hydroxylase positive (TH1⁺; mainly dopaminergic), and serotonergic (5-HT⁺) axons and cells during successful spinal cord regeneration in adult zebrafish. TH1⁺ axons in the spinal cord are exclusively derived from the brain. After spinal cord transection, TH1⁺ immunoreactivity is completely lost from the caudal spinal cord. Axons increase in density rostral to the lesion site compared to unlesioned controls and re-innervate the caudal spinal cord at 6 weeks post-lesion. Interestingly, axons mostly fail to regrow to more caudal levels of the spinal cord even after prolonged survival times. However, axon regrowth correlates with recovery of swimming behavior, which is completely lost again after re-lesion of the spinal cord. Similar observations were made for descending 5-HT⁺ axons from the brain. In addition, spinal 5-HT⁺ neurons were newly generated after a lesion and transiently increased in number up to 5-fold, which depended in part on hedgehog signaling. Overall, TH1⁺ and 5-HT⁺ innervation is massively altered in the successfully regenerated spinal cord of adult zebrafish. Despite these changes in TH and 5-HT systems, a remarkable recovery of swimming capability is achieved, suggesting significant plasticity of the adult spinal network during regeneration.

INTRODUCTION

Adult zebrafish, in contrast to mammals, are capable of successful spinal cord regeneration, such that plastic changes in cellular and axonal compartments that are associated with functional regeneration can be studied in this genetically tractable vertebrate (Becker and Becker, 2007). We have previously shown that the number of supraspinal neurons that grow axons beyond a spinal lesion site correlates with recovery of swimming capability (Becker et al., 1997). Moreover, when regeneration over a spinal lesion site in goldfish (Bernstein and Bernstein, 1969) or zebrafish is mechanically blocked, no recovery occurs. The same is true when regrowth of descending axons is selectively compromised by knock down of L1.1, a cell recognition molecule necessary for axon regrowth (Becker et al., 2004). Thus axon regrowth beyond the lesion site is crucial for recovery from a spinal lesion. However, recovery is seen in eel (Doyle et al., 2001) and lamprey (McClellan, 1994) already when axons have only grown for a short distance into the distal cord, indicating that the spinal circuitry can produce swimming patterns without complete re-innervation of the distal cord.

In addition, a lesion induces neurogenesis in the spinal cord of teleost fish (for recent review, see Sirbulescu and Zupanc, 2010), including zebrafish (Reimer et al., 2008). Elucidating the changes that specific axonal and cellular systems undergo during regeneration on a quantitative basis may help to gauge the magnitude of overall plasticity that leads to the remarkable functional recovery in adult zebrafish after a spinal lesion.

We decided to study changes in the dopaminergic and serotonergic systems, because both of these have been shown to critically influence the output of the spinal central pattern generator for locomotion (reviewed in Jordan et al., 2008; Grillner and Jessell, 2009). In larval zebrafish dopaminergic innervation of the spinal cord is

derived exclusively from the diencephalon (McLean and Fetcho, 2004; Kastenhuber et al., 2010). Dopamine suppresses initiation of swimming in pre-feeding larvae (Thirumalai and Cline, 2008) and the agonist clozapine causes hypoactivity in older embryos (Boehmler et al., 2007). In larval lampreys dopamine inhibits glutamatergic reticulospinal transmission (Svensson et al., 2003) and increases or decreases fictive swimming frequency in a dose-dependent manner (McPherson and Kemnitz, 1994).

Serotonergic innervation of the spinal cord originates from supraspinal raphe neurons (Lillesaar et al., 2009) and from spinal intrinsic neurons (van Raamsdonk et al., 1996) in adult zebrafish. Endogenous serotonin decreases the frequency of NMDA induced locomotor rhythms in fictive swimming of adult zebrafish spinal cord preparations by increasing mid-cycle inhibitory postsynaptic potentials and delaying the subsequent onset of hyperpolarization (Gabriel et al., 2009). Moreover, serotonin has been shown to directly modulate firing patterns of rat motor neurons (Kjaerulff and Kiehn, 2001).

Previous work has presented evidence for regeneration of dopaminergic and serotonergic axons in the spinal cord of zebrafish (van Raamsdonk et al., 1998a), regeneration of serotonergic descending axons in the spinal cord of the sea lamprey (Cornide-Petronio et al., 2011), and serotonergic cells have been shown to be generated in the lesioned spinal cord of the closely related goldfish (Takeda et al., 2008). However quantitative analyses of axon densities necessary for a comparison of the regenerated and the unlesioned situation are missing. We find that both dopaminergic and serotonergic systems undergo massive changes after a spinal lesion, with several anatomical parameters not returning to levels observed in unlesioned animals even after prolonged survival times. These changes include hyper-innervation of the rostral spinal cord, reduced re-innervation of the caudal spinal cord and a lack of regrowth to more caudal levels for both systems. This

suggests that considerable plasticity of the locomotor network occurs to lead to recovery of swimming function.

MATERIAL AND METHODS

Animals

All fish are kept and bred in our laboratory fish facility according to standard methods (Westerfield, 1989) and all experimental procedures have been approved by the British Home Office. We used wild type (wik), tg(*olig2*:GFP) (Shin et al., 2003) and tg(*shha*:GFP) (Shkumatava et al., 2004).

Spinal lesion and intraperitoneal injections of substances

Lesions were performed as previously described (Becker et al., 1997). Fish were anesthetized in 0.033% aminobenzoic acid ethyl methyl ester (MS222; Sigma). A longitudinal incision was made at the side of the fish to expose the vertebral column. The spinal cord was completely transected 3.5 mm caudal to the brainstem-spinal cord junction under visual control.

Bromodeoxyuridine (BrdU, B9285, Sigma) was injected intraperitoneally at a concentration of 5 mg/ml in a volume of 25 µl PBS (pH 7.4) at 0, 2 and 4 days post-lesion (dpl), as described (Reimer et al., 2008). Analysis took place at 2 or at 6 weeks post-lesion (wpl)

Cyclopamine and the chemically related control substance tomatidine were injected at 10 mg/kg bodyweight at 3, 6, and 9 dpl, as previously published (Reimer et al., 2009). Analysis took place at 42 dpl.

Immunohistochemistry and in situ hybridization

We used a number of primary antibodies. Rat anti-BrdU (1:500, 0BT0030CX, Serotec, Munich, Germany) was specific, because it labels cell nuclei only after prior incubation with BrdU. Chicken anti-GFP (1:500, ab13970, Abcam,

Cambridge, UK) labels cellular structures only in GFP transgenic animals indicating specificity of labeling. Mouse anti-TH1 (1:1000, MAB318, Millipore, CA, USA) has been shown to specifically label TH1 in zebrafish (Chen et al., 2009) and immunolabeling in embryos is abolished after anti-sense morpholino mediated knock down (data not shown), strongly suggesting specific labeling. We have previously shown the specificity of rabbit anti-dopamine (1:750, H.W.M. Steinbusch, University of Maastricht) in immunohistochemistry in the central nervous system (Barreiro-Iglesias et al., 2008, 2009) and colocalization with TH1 immunoreactivity supported the specificity of labeling (present results). Specificity of the mouse anti-5HT antibody (1:1000, MAB318/LNC1, Millipore, Billerica, MA, USA) in immunohistochemistry has been shown (Olsson et al., 2008). Mouse anti-SV2 (synaptic vesicle, 1:20, Developmental Studies Hybridoma Bank, University of Iowa, IO, USA) is a standard antibody to label synaptic specializations in zebrafish (for example, Jonz and Nurse, 2003; Neill et al., 2004; Jing et al., 2009). Goat anti-synaptophysin (SYP(c-20), 1:50, sc 7568, Santa Cruz Biotechnology) labels in a punctuate pattern only in synaptic areas, which is very similar to that of SV2, suggesting specific labeling. Secondary Cy2-, Cy3- and Cy5-conjugated antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, USA).

Immunohistochemistry on 50 μ m vibrating blade microtome sections have been described (Reimer et al., 2009). To detect synaptophysin, sections were incubated with the antibody for 48 hours instead of overnight. For simultaneous detection of dopamine and TH1 primary and secondary antibodies were simultaneously applied and a modified protocol was used for primary antibody incubation: floating sections of spinal cords (50 μ m in thickness) of perfusion-fixed animals (2% paraformaldehyde / 2.5% glutaraldehyde, 1% metabisulfite) were washed in phosphate buffered saline/1% metabisulfite (pH adjusted to 7.4) with 0.1%

Triton X-100 (PBS Mb Tx) for two times 15 min. Then, sections were incubated in 0.1% NaBH₄ in ddH₂O for 30 min followed by two washing steps of 15 min each with PBS Mb Tx. Sections were blocked in 1.5% normal goat serum in PBS Mb Tx PBS for 30 min. Then, primary antibodies were diluted in PBS Mb Tx and incubated with the sections overnight at 4°C. Secondary antibody incubation followed the standard protocol. For documentation and analysis, we used either a Zeiss LSM 510 or a Zeiss 750 LSM Confocal Microscope using 20x and 63x oil lenses.

The method for non-radioactive in situ hybridization on 50 µm vibrating blade microtome sections (Lieberoth et al., 2003) and the plasmids to generate probes for *th2* (Filippi et al., 2010) have been described.

Retrograde axonal tracing combined with immunohistochemistry

Retrograde tracing of brain neurons with rhodamine dextran amine (RDA) or biocytin has been described (Becker et al., 1997). Briefly, a piece of re-crystallized RDA or biocytin was applied to the completely transected spinal cord and allowed to be transported over night (biocytin) or 6 days (RDA). Animals were perfusion fixed, brains were dissected and sectioned on a vibrating blade microtome and processed for immunohistochemistry as described above. The RDA and antibody signals were then detected by fluorescence microscopy.

Test of swimming capability

As previously described, we tested the swimming capability of lesioned fish by determining the time they were able to hold their position in a water current (Reimer et al., 2009). Recovery was assumed after 1 h in a current of 7cm/s.

Axon and cell quantification

For axon quantifications we used a Zeiss LSM 510 Confocal Microscope using a 20x Apochromate 0.75 lens for acquiring images. TH1⁺ or 5-HT⁺ profiles were quantified applying the Feature J Hessian plug-in for Image J (smallest eigenvalue Hessian tensor; smoothing scale to 1.0; <http://rsbweb.nih.gov/ij/plugins/index.html>) on maximum projection z-stacks (15 optical sections, thickness 1.5 µm) of whole spinal 50 µm sections according to an established protocol (Grider et al., 2006). Profiles were quantified in binary images (threshold 140) using the “analyse particle” function of Image J with particle size 1 to infinity and circularity from 0.00 to 1.00. Comparing this method with manual counts indicated an average divergence of 6.25%. Images that contained fewer than approximately 100 profiles were manually counted.

Numbers of serotonergic cells were determined from stereological counts of confocal images of the three randomly selected 50 µm thick vibratome sections from the region up to 750 µm rostral to the lesion site and three sections 750 µm caudal to the lesion site. Numbers were calculated separately rostral and caudal to the lesion site and then summed for cell numbers in the entire 1.5 mm surrounding the lesion site.

Statistical analysis

For statistical analysis, the program Prism (GraphPad Software, La Jolla, CA, USA) was used. Variability of values is given as SEM. Statistical significance was determined by using Mann-Whitney-U test for single comparisons or ANOVA followed by Dunnet's or Tukey's post test for multiple comparisons.

RESULTS

The aim of this study was to quantify the plastic changes in dopaminergic and serotonergic systems during successful spinal cord regeneration in an adult vertebrate.

Spinal dopamine is exclusively derived from the brain.

To localize dopamine signals in the adult spinal cord, we analyzed the expression of TH1 and TH2, the rate limiting enzymes for dopamine synthesis. Immunohistochemistry with a TH1 specific antibody (Chen et al., 2009) at a midthoracic level (~3.5 mm caudal to the brainstem/spinal cord transition) and at the level of the dorsal fin (~7 mm caudal to the brainstem/spinal cord transition) in unlesioned fish revealed circuitous axons with numerous protrusions, many of which were double-labeled with the synaptic marker synaptophysin (Fig. 1A,B). No TH1⁺ cell bodies were found in the spinal cord (Fig. 1A). Double immunohistochemistry with the dopamine antibody revealed that more than 80% of the TH1⁺ axons were dopaminergic, indicating that TH1⁺ axons in the spinal cord are mainly dopaminergic (Fig. 1A,C). The remaining TH1⁺ profiles could be noradrenergic axons possibly originating from the locus coeruleus as previously described at early developmental stages (McLean and Fetcho, 2004; Kastenhuber et al., 2010).

In situ hybridization for TH2 indicated the absence of TH2 expressing cells in the spinal cord. However, labeled cell bodies were found in the posterior periventricular preoptic nucleus, which projects to the spinal cord (Fig. 1E) (Becker et al., 1997). In agreement with a minor contribution of TH2⁺ axons to the dopaminergic innervation of the spinal cord, $10.4 \pm 3.19\%$ ($n = 2$ animals) of spinal axons that were labeled by a dopamine antibody were TH1⁺.

To localize the source of TH1⁺ axons in the brain, we retrogradely traced the descending projection from a midthoracic level in combination with TH1 immunohistochemistry (Fig. 1D). This showed double-labeled cells in the dopaminergic periventricular nucleus of the posterior tuberculum in the diencephalon only, similar to what has been reported for the embryonic spinal cord (McLean and Fetcho, 2004; Kastenhuber et al., 2010). Overall, this suggests that the major source of dopamine in the spinal cord are TH1⁺ axons coming from the brain and that TH1⁺ axons are mostly dopaminergic. No spinal intrinsic cellular source of dopamine was detectable.

Lesion-induced quantitative changes in TH1[±] axons in the spinal cord.

We examined the time course of changes in TH1⁺ axon numbers “rostral” (within 750 μ m), “caudal” (within 750 μ m) and “far caudal” (3500 μ m) to the position of a midthoracic lesion site (Fig. 2A) at 1, 2, 6 and 13 wpl. We chose these time points, because Wallerian degeneration has occurred to severed axons in the caudal spinal cord at 1 wpl, axon regrowth beyond the lesion site starts at around 2 wpl, and functional recovery plateaus at 6 wpl (Becker et al., 2004). We chose the 13 wpl time point to detect any late changes in innervation. For the late time points (6 and 13 wpl), when recovery can be assessed, values are only given for animals that showed recovery in a forced swim test, if not stated otherwise.

Rostral to the lesion site, TH1⁺ axon profile numbers showed a non-significant reduction at 1 wpl (unlesioned, 673.2 ± 52.70 profiles/section, $n = 7$; 1 wpl: 391.4 ± 82.43 , $n = 5$, $P > 0.05$). Thereafter a continuous and significant increase was observed to at least 13 wpl (2wpl: 952.6 ± 85.82 profiles/section, $n = 6$; 6wpl: 1204.6 ± 145.36 , $n = 6$ 13wpl: 1571.9 ± 368.55 , $n = 4$), the last time point examined, to levels

that were 2.3 fold increased over those in unlesioned fish. This time course is consistent with initial die-back and later sprouting of axons (Fig. 2 B,C,D).

Caudal to the lesion site, almost no axons were observed at 1 wpl (0.9 ± 0.61 profiles/section, $n = 5$), consistent with a supraspinal origin of these axons and their subsequent Wallerian degeneration. Some axons were visible at 2 wpl (24.8 ± 10.32 profiles/section, $n = 6$) and at 6 wpl a significant increase in axon profile numbers (296.3 ± 80.57 profiles/section, $n = 6$; $P < 0.01$;) was observed, compared to 1 wpl. This indicated regrowth of TH1⁺ axons beyond the lesion site. At 13 wpl, axon profile numbers appeared reduced again (149.3 ± 124.35 profiles/section, $n = 4$; Fig. 2E,F), which was, however, not statistically significant. Thus, immediately caudal to the lesion site the number of TH1⁺ axon profiles was restored to maximally 44% of that in unlesioned animals at 6 wpl.

Far caudal to the midthoracic position of the lesion site, unlesioned animals showed substantial TH1⁺ innervation (484.6 ± 86.01 axon profiles/section, $n = 4$). This had disappeared by 1 wpl (1wpl: 1.7 ± 1.65 profiles/section, $n = 5$). Surprisingly, no appreciable reinnervation at this position was observed for up to 13 wpl (2 wpl: 2.8 ± 2.45 profiles/section, $n = 3$; 6 wpl: 3.3 ± 3.33 , $n = 6$; 13 wpl: 6.5 ± 2.77 , $n = 4$; Fig. 2G,H,I). This indicated that regenerating TH1⁺ axons did not reach previously innervated far caudal levels.

TH1⁺ axon regrowth correlates with recovery of swimming capability.

As previously reported, regenerative success in spinal lesioned fish is variable and anatomical regeneration of axons beyond the lesion site correlates with regenerative success (Becker et al., 1997; Becker et al., 2004). We wanted to determine whether plastic changes of TH1⁺ axons also correlate with recovery of function. To assess recovery of swimming capability we used a previously

established test of forced swimming against a water flow (Fig. 3A) (Reimer et al., 2009). In a set of 37 fish, we found that 17 fish were unable to hold their position in the water flow for more than 30 seconds. These fish were classified as non-recovered. Another 17 fish held their position for an hour, at which time the test was terminated. These fish were classified as recovered. Unlesioned fish always sustained swimming to one hour. Three individuals that showed intermediate swim times (9, 13 and 50 minutes) were also classified as recovered (Fig. 3B). Comparing TH1⁺ axon numbers rostral to the lesion site between recovered and non-recovered fish did not show differences at 6 wpl (recovered: 1204.6 ± 145.36 profiles/section, $n = 6$; non-recovered: 1011.4 ± 115.82 , $n = 6$, $P > 0.05$; Fig. 4A,B). In contrast, non-recovered fish showed significantly less axonal regrowth caudal to the lesion site compared to recovered fish (recovered: 296.3 ± 80.57 profiles/section, $n = 6$, non-recovered: 39.7 ± 32.31 , $n = 6$; $P < 0.05$; Fig. 4C,D). Thus growth of TH1⁺ axons beyond the lesion site correlates with recovery of swimming capability.

Re-lesion abolishes functional recovery and TH1[±] axons caudal to the lesion site.

To determine whether recovery of swimming capability and TH1⁺ innervation of the caudal spinal depend on continuity of the spinal cord we re-lesioned the spinal cord in the same position in three recovered animals that held their position in the water flow for 60 minutes. All three animals completely lost their swimming capability (Fig. 3C) and TH1⁺ axons were almost completely absent caudal to the lesion site (0.4 ± 0.43 profiles/section, $n = 3$) 1 week after re-lesion. Thus functional recovery is unlikely to be due to plasticity of spinal circuitry caudal to the lesion site alone and TH1⁺ re-innervation of the caudal spinal cord after a lesion is most likely derived from descending axons that regenerated beyond the spinal lesion site.

Lesion-induced quantitative changes in 5-HT axons in the spinal cord.

Using a 5-HT antibody we detect circuitous axons with numerous protrusions as well as cell bodies in the spinal cord at a midthoracic level (Fig.5A), and at the level of the dorsal fin (Fig. 6F). Many of the axonal protrusions were double-labeled with the synaptic marker SV2 (Fig. 5B). Using retrograde tracing from a midthoracic level in combination with 5-HT immunohistochemistry we find double-labeled cells in the inferior raphe region of the brainstem (Fig. 5C,D), confirming a previous report of 5-HT⁺ neurons with descending axons in this region in adult zebrafish (Lillesaar et al., 2009). Thus 5-HT⁺ axons in the spinal cord are of supraspinal and local origin.

We established a time course for changes in the density of 5-HT⁺ axon profiles after a spinal lesion in the same animals used for TH1 immunohistochemistry, assessing the same locations and time points. Rostral to the lesion site, the number of axonal profiles was non-significantly reduced at 1 wpl (unlesioned: 679.1 ± 126.32 profiles/section, $n = 7$; 1 wpl: 505.8 ± 36.95 , $n = 5$; $P > 0.05$). Thereafter, numbers increased until they were 80% higher than in unlesioned animals at 6 wpl (2 wpl: 989.8 ± 155.38 , $n = 6$; 6wpl: 1224.1 ± 105.04 profiles/section, $n = 6$, $P < 0.05$). Numbers appeared slightly reduced again at 13 wpl (1048.2 ± 247.04 profiles/section, $n = 4$; Fig. 6A-C). This suggests considerable sprouting of 5-HT⁺ axons rostral to the lesion site.

Caudal to the lesion site, the number of axon profiles was strongly and significantly reduced at 1 wpl (35.0 ± 5.62 profiles/section, $p < 0.001$) compared to unlesioned control animals. As opposed to TH1⁺ axons, some axons remained at 1 wpl, suggesting that these originated from local 5-HT⁺ neurons, whereas descending axons had undergone Wallerian degeneration at this time point. Numbers of axonal profiles significantly increased thereafter and peaked at 6 wpl (2 wpl: 103.0 ± 17.41

profiles/section, 6 wpl: 383.7 ± 71.91 , $n = 6$). The increase in axon profile numbers between 1wpl and 6 wpl was statistically significant ($P < 0.05$). At 13 wpl (132.1 ± 19.42 profiles/section, $n = 3$), there was a non-significant reduction in profile numbers, compared to 6 wpl (Fig. 6D,E). Thus axon numbers gradually increased again caudal to a lesion site to maximally 58% of the number in unlesioned animals at 6 wpl.

Far caudal to the lesion site, we also observed a huge reduction in axon profile numbers at 1wpl (unlesioned: 659.9 ± 110.05 profiles/section, $n = 3$; 1 wpl: 32.5 ± 7.86 , $n = 5$, $p < 0.001$). Although numbers of axons were slightly increased again at 2 wpl (67.8 ± 5.31 profiles/section, $n = 3$) and 6 wpl (91.3 ± 14.23 profiles/section, $n = 6$), none of these changes were statistically significant. Even at 13 wpl (64.2 ± 17.85 profiles/section, $n = 3$) no significant increase in profile number was observed (Fig. 6F-H). This is consistent with descending 5-HT axons not regrowing to far caudal levels.

Number of 5-HT[±] axons caudal to the lesion site correlates with recovery of swimming capability

Rostral to the lesion site there was no significant difference between recovered (1224.1 ± 105.04 profiles/section, $n = 6$) and non-recovered fish (966.8 ± 70.95 profiles/section, $n = 6$, $P > 0.05$) in the number of 5-HT⁺ axon profiles at 6 wpl (Fig. 7A,B). In contrast, caudal to the lesion site recovered fish (383.7 ± 71.91 profiles/section, $n = 6$) had significantly more axonal profiles than non-recovered ones (88.0 ± 24.12 profiles/section, $n = 6$; $P < 0.01$; Fig. 7C,D). This suggests that regrowth of 5-HT⁺ axons beyond a spinal lesion is associate with recovery of swimming capability.

The contribution of endogenous spinal neurons to spinal innervation increases after a lesion

By comparing axon profile numbers at 1 wpl (~35 profiles/section) with the unlesioned situation (~679 profiles/section) we estimated that local 5-HT cells make a minor contribution (~5%) to spinal 5-HT innervation in unlesioned animals. After re-lesion at 6 wpl and analysis 1 week after re-lesion, 122.1 ± 32.95 profiles/section ($n = 3$) profiles were observed (Fig. 7C,D). This was a substantial reduction compared to ~384 profiles/section observed at 6 wpl, indicating loss of descending axons that had regenerated over the lesion site. Importantly, this also demonstrated a 3.5-fold increase in the number of spinal-derived 5-HT⁺ axons after regeneration (1 wpl vs. 1 week after re-lesion: $P < 0.036$). Increased innervation by spinal 5-HT⁺ neurons is probably a consequence of lesion-induced generation of 5-HT⁺ neurons, as observed in goldfish (Takeda et al., 2008).

Lesion-induced generation of 5-HT⁺ cells after a lesion.

Numbers of 5-HT⁺ cells did not differ significantly between the rostral and caudal level at the different time points and are therefore presented together. Absolute numbers of 5-HT⁺ cells increased steadily after a lesion up to 6 wpl (unlesioned: 93.1 ± 14.17 cells/1500 μm spinal cord, $n = 18$; 1 wpl: 120.0 ± 41.13 , $n = 5$; 2 wpl: 363.4 ± 30.41 , $n = 19$; 6 wpl: 471.2 ± 80.57 , $n = 10$; Fig. 8A,B) when the number of 5-HT⁺ cells was 5.1-fold higher than in unlesioned animals ($P < 0.001$). At 13 wpl (217.8 ± 16.70 cells, $n = 5$) a significant reduction in cell number was observed compared to 6 wpl ($P < 0.05$). This number was not significantly different from that in unlesioned animals anymore. Far caudal to the lesion site, no significant changes in cell numbers occurred (unlesioned: 200 ± 22.21 cells/1500 μm , $n = 3$; 1wpl: 137 ± 30.95 , $n = 5$, 2wpl: 163.6 ± 29.30 , $n = 3$; 6wpl: 229.6 ± 35.68 , $n = 6$; 13

wpl: 100.0 ± 34.55), in agreement with previous observations that proliferation in the ventricular progenitor zone is much lower at distant levels (Reimer et al., 2008).

Overall, this suggests an overproduction of 5-HT⁺ cells close to the lesion site that is later pruned back.

To directly show that 5-HT⁺ neurons were newly generated after the lesion, we injected animals with the proliferation marker BrdU at 0, 2, and 6 days post-lesion. This labeled 74.8 ± 21.10 5-HT⁺/BrdU⁺ cells ($n = 8$) at 2 wpl and 209.8 ± 26.93 cells ($n = 8$) at 6 wpl. No cells were double-labeled in unlesioned fish ($n = 3$; Fig. 8C,D). Even though we presumed that those 5-HT⁺ cells in lesioned animals that exceeded the number in unlesioned fish were newly-generated, not all of them were double labeled with BrdU. This was expected, because our BrdU injection scheme also labels only ~25% of newly generated motor neurons after a lesion (Reimer et al., 2008).

We noticed that 5-HT⁺ cells contacted the central canal only in the lesioned spinal cord at 6 and 13 wpl, suggesting that these were recently generated at the central canal. The central canal is lined with the spinal progenitor cells which are subdivided into dorso-ventral expression domains of transcription factors that are similar to those that give rise to different cell types during embryonic development (Reimer et al., 2008). We find that 5-HT⁺ cells contacting the central canal are not randomly distributed. By double labeling of 5-HT in Olig2:GFP transgenic fish, we find that all central canal contacting 5-HT⁺ cells contact the central canal either ventral to the olig2:GFP⁺ zone ($n = 12$ cells; Fig. 9B) or in the olig2:GFP zone itself ($n = 5$ cells). In Shh:GFP transgenic animals, two 5-HT⁺ cells were observed in close proximity to Shh:GFP⁺ ependymo-radial glial cells and five cells appeared to be intermingled with these cells, but were always Shh:GFP negative (Fig. 9A). However, 5-HT was never co-labeled with Olig2:GFP or Shh:GFP. This distribution of central

canal-contacting 5-HT neurons is consistent with a ventral progenitor zone for 5-HT⁺ neurons after a lesion, comprising the Olig2:GFP⁺ zone and a more ventral zone.

Blocking hedgehog activity reduces the number of 5-HT⁺ neurons

The ventral origin of 5-HT neurons after lesion suggests an involvement of ventral ependymo-radial glia derived hedgehog signaling, which has been shown to promote regeneration of motor neurons after a spinal lesion (Reimer et al., 2009). To test this, we used intraperitoneal injections of the Smoothed antagonist cyclopamine, previously shown to reduce motor neuron generation and expression of the hedgehog target gene *patched1* in motor neuron progenitor cells (Reimer et al., 2009). Analysis took place at 6 wpl, when the number of newly generated 5-HT⁺ neurons peaked.

Numbers of 5-HT⁺ cells after cyclopamine treatment were significantly reduced compared to fish injected with a related control substance, tomatidine, by 23% (tomatidine: 10.8 ± 0.86 cell profiles/50 μm , $n = 8$; cyclopamine: 8.3 ± 1.00 cell profiles/50 μm ; Mann-Whitney-U, one tailed, $p = 0.0406$). This suggests that shh promotes generation of 5-HT⁺ cells in the lesioned spinal cord.

Numbers of newly generated 5-HT⁺ neurons caudal to the lesion site correlate with recovery of swimming capability and axon regrowth.

To determine whether numbers of 5-HT⁺ cells correlate with recovery of swimming capability, we compared numbers of 5-HT⁺ cells between recovered and non-recovered fish at 6 wpl, when cell numbers peak and regenerative success can be assessed. Rostral to the lesion site, differences in 5-HT⁺ cell numbers were not significant between recovered (365.0 ± 73.59 cells/750 μm , $n = 6$) and non-recovered fish (292.6 ± 63.14 cells/750 μm , $n = 6$; $P > 0.05$; Fig. 10A,B). In contrast, caudal to

the lesion site, fewer 5-HT⁺ cells were present in non-recovered (111.1 ± 18.59 cells/750 μ m) vs. recovered fish (253.7 ± 28.32 ; $P = 0.0198$; Fig. 10A,C), suggesting some interaction between re-growing axons and newly-generated 5-HT⁺ cells.

DISCUSSION

We show for the first time that the TH1 and 5-HT systems are substantially altered in spinal lesioned zebrafish concomitant with recovery of swimming function (summarized in Fig. 11). Descending axons sprout rostral to the lesion site and reinnervate levels beyond the lesion site for only a short distance, re-establishing about half of the previous density of axons. Far caudal positions are not re-innervated. Despite this relatively weak regenerative growth, axon growth beyond the lesion site strictly correlates with recovery of function. Spinal-intrinsic 5-HT⁺ neurons are first over-produced in the ventral spinal cord in a hedgehog dependent manner and later pruned back to numbers that are comparable to those in unlesioned animals.

For both TH1⁺ and 5-HT⁺ descending axons, growth over the lesion site is associated with functional recovery. Importantly, re-lesioning recovered fish abolishes TH1⁺ and 5-HT⁺ descending axons caudal to the lesion site as well as recovered swimming function. This is in agreement with earlier studies showing that fish in which axon regrowth is sparse or experimentally reduced failed to recover (Becker et al., 1997; Becker et al., 2004). While this suggests that regrowth of TH1⁺ and 5-HT⁺ descending axons may be important for recovery of the fish, pre-lesion patterns are not restored. Both monoaminergic systems studied here regrow only about half of the axons previously observed directly caudal to the lesion site. Moreover, more caudal levels of the spinal cord are mostly not re-innervated. A previous study also reported regrowth of descending TH1⁺ and 5-HT⁺ axons in spinal-lesioned zebrafish (van Raamsdonk et al., 1998b). However these limitations were not noticed, due to a lack of quantitative analyses. That study found axon regrowth to 6 mm caudal to the lesion site. We also find long distance regrowth,

which was however exceptional, as it was observed in only one out of 6 recovered fish.

What are the reasons for limited regrowth of TH1⁺ and 5-HT⁺ descending axons? Extrinsic factors, such as a glial scar (Busch and Silver, 2007) or myelin inhibitors (Schwab, 2004) as in mammals are unlikely reasons, because no evidence has been found for either of these in fish (Nona and Stafford, 1995; Becker and Becker, 2002; Abdesselem et al., 2009). Moreover, up to half of the TH1⁺ and 5-HT⁺ axons manage to regrow into the caudal spinal cord and many other brain nuclei regenerate axons even into the distal spinal cord (Becker et al., 1997). It is more likely that the axotomy was too distal from the neuronal somata of TH1⁺ and 5-HT⁺ neurons to elicit a robust regenerative response in descending TH1⁺ and 5-HT⁺ axons. Indeed, some brain nuclei show a regenerative response, including upregulation of growth-related genes, only when axotomy occurred close to the soma, i.e. after a more rostral spinal lesion (Becker et al., 1998). This is similar to mammalian neurons (Doster et al., 1991; Tetzlaff et al., 1994). In support of this explanation, we found robust growth of TH1⁺ and 5-HT⁺ axons 3.5 mm into the spinal cord in one fish that was lesioned at the brainstem/spinal cord junction (data not shown). Interestingly, 5-HT⁺ axons have superior sprouting capacity compared to other axon types in mammals, demonstrated by their ability to sprout and penetrate a glial scar after injury (Hawthorne et al., 2011). Thus, in our lesion model TH1⁺ and 5-HT⁺ cells might not have initiated a significant regenerative response after a lesion at the more distal mid-thoracic level.

Our observations open up the opportunity to learn how successful recovery of function can be achieved with limited anatomical regeneration of specific systems. Apparently, re-innervation of far caudal spinal segments by TH1⁺ and 5-HT⁺ descending axons is not necessary for recovery of substantial swimming function. It

is possible that the significantly sprouted axons rostral to the lesion site as well as those axons that did reinnervate the caudal cord are capable of modulating the propagating rostro-caudal wave of excitation in the spinal network during swimming (Grillner and Wallen, 2007) without the need to do this at every level of the spinal cord. Alternatively, deficits in function that may be associated with altered TH1⁺ and 5-HT⁺ innervation of the spinal cord might not have been revealed by the functional test of swimming endurance applied here.

Another way in which a lack of long distance TH1⁺ and 5-HT⁺ descending axons could be compensated for is plasticity of the intraspinal circuitry caudal to the lesion site. For example, spinal intrinsic neurons, which are located at some distance caudal to a lesion site and have not been axotomized by the lesion, up-regulate expression of the growth-associated protein 43 and the cell recognition molecule L1.1 after a spinal lesion (Becker et al., 2005). Both of these molecules are indicators of neuronal plasticity (Benowitz and Routtenberg, 1997; Kapfhammer, 1997; Emery et al., 2003). Similarly, the rat motor cortex shows plastic changes after ablation of dopaminergic input (Viaro et al., 2011).

Could the loss of descending 5-HT⁺ axons be offset by newly-generated spinal 5-HT⁺ neurons or axonal sprouting of local 5-HT⁺ neurons? Indeed, there is massive generation of 5-HT cells in the ventral spinal cord, far exceeding the number of cells in unlesioned animals. However, this increase occurs only in the vicinity of the spinal lesion site and cell numbers are later pruned back to a level that is not significantly elevated anymore over that in unlesioned animals. Sprouting of 5-HT⁺ spinal neurons in the caudal spinal cord that is denervated of descending 5-HT⁺ input could also compensate for a lack of descending input. However, far caudal to the lesion site (not reached by regenerating descending axons), axon numbers never significantly increase, despite prolonged (13 weeks) depletion of 5-HT⁺ axons. Thus neither

neurogenesis nor axonal sprouting of spinal-intrinsic 5-HT⁺ cells compensates for lost descending 5-HT⁺ axons.

Adult zebrafish regenerate spinal 5-HT⁺ neurons in a hedgehog dependent manner. Our analysis has shown that only during regeneration, radially elongated 5-HT neurons contacted the ventricle in adult animals. This is similar to newly generated motor neurons shown to have ventricular contact during spinal cord regeneration (Reimer et al., 2008). Given that lesion-induced proliferation of progenitor cells occurs at the ventricle this provided us with the opportunity to estimate where 5-HT⁺ cells potentially originate. In contrast to motor neurons, which are born in a narrow olig2:GFP⁺ zone of ependymo-radial progenitor cells, 5-HT⁺ cells have a wider ventral region of origin. Nevertheless, regeneration of this cell type appears to be promoted by a ventral midline derived hedgehog signal similar to motor neurons (Reimer et al., 2009), as determined by blocking this signal with cyclopamine, a strong and specific antagonist of the Hedgehog receptor Smoothened. It is important to note that generation of 5-HT⁺ neurons (this report) and motor neurons (Reimer et al., 2008), as well robust hedgehog signaling is only detectable in the lesioned spinal cord (Reimer et al., 2009). This is different from neurogenic zones in the brain of zebrafish (Adolf et al., 2006; Kaslin et al., 2009) and mammals (reviewed, for example in Gould, 2007) that are constitutively active. In the unlesioned spinal cord of zebrafish and mammals, progenitor cells are quiescent (Meletis et al., 2008). However, the lesioned adult spinal cord of zebrafish regenerates different cell types in a hedgehog-dependent manner. Therefore, finding the switch that allows neurogenesis in the lesioned spinal cord of adult zebrafish is a future goal.

Some changes in the TH1⁺ and 5-HT⁺ systems occur at late stages of regeneration, between 6 and 13 weeks post-lesion, i.e. after functional recovery was

achieved. Most remarkable is the significant reduction in the number of 5-HT⁺ neurons. This could be due to limited trophic support of these cells or due to feedback from a re-arranged spinal circuitry. While in the closely related goldfish, a constant increase in the number 5-HT neurons after a spinal lesion was also noted, no reduction was seen at late stages (Takeda et al., 2008). It is possible that this phenomenon only occurs after the last time point analyzed in goldfish. Interestingly, numbers of descending TH1⁺ and 5-HT⁺ axons regenerated beyond the lesion site are also slightly reduced again at 13 wpl compared to 6 wpl, whereas the sprouted axons rostral to the lesion site remained. This supports the notion that considerable plasticity is occurring in the caudal lesioned spinal cord even after recovery of swimming function.

Overall our study supports that the lesioned spinal cord of adult zebrafish undergoes substantial axonal and cellular plasticity after a lesion, which leads to functional recovery. Significant alterations in TH1⁺ and 5-HT⁺ systems, including a lack of regrowth to distal levels of the spinal cord reveals that for successful spinal cord regeneration, re-establishing the pre-lesion circuitry is not a prerequisite. However, this and previous studies highlight the importance of axon regrowth over the lesion site for recovery to occur.

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FIGURES

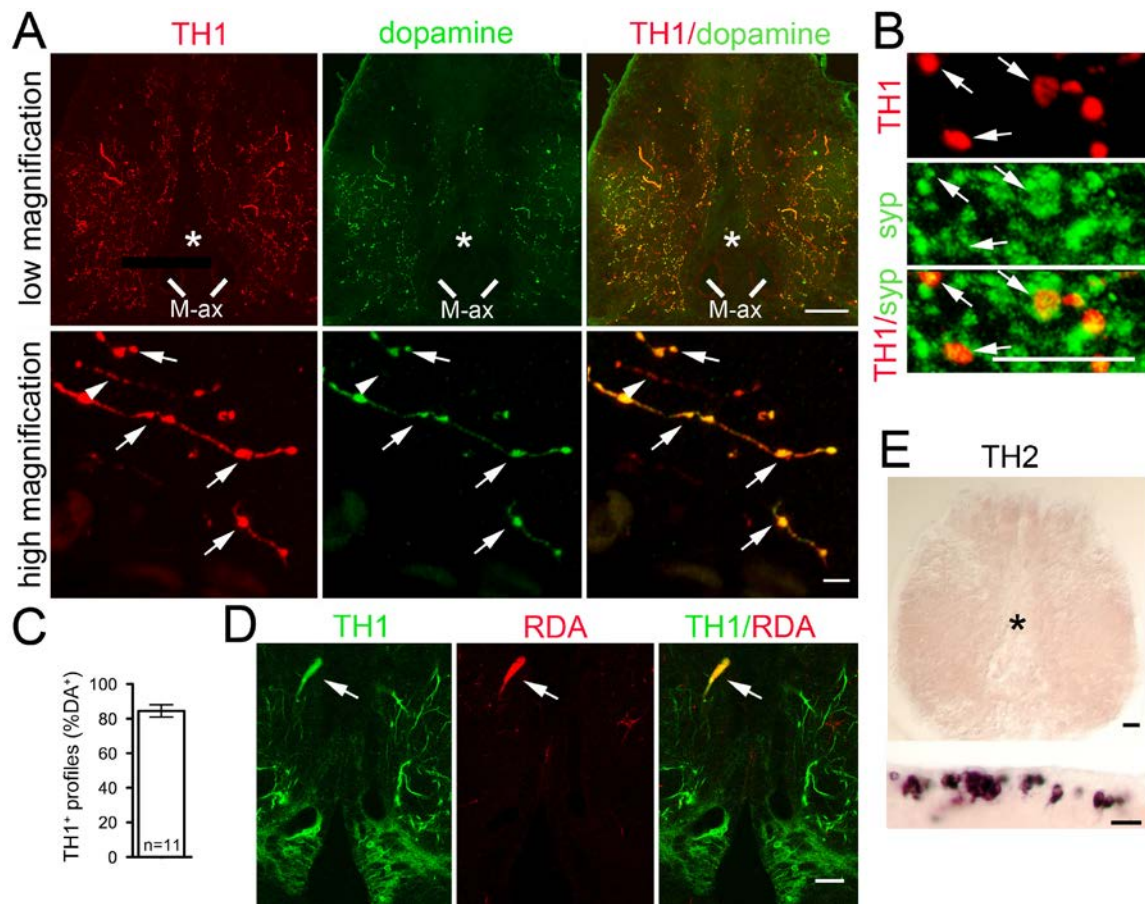


Fig. 1 TH1⁺ axons in the spinal cord are mostly dopaminergic and TH1⁺ and TH2⁺ cells are located only in the brain. **A:** Double-labeling of TH1 and dopamine in spinal cross sections at a mid-thoracic level reveals overlap (arrows). An axon labeled only by the TH1 antibody (arrowhead), the central canal (asterisk), the Mauthner axons (M-ax) are indicated. **B:** TH1⁺ protrusions double-label with synaptophysin (syp; arrows). **C:** Quantification of TH1⁺ axons double-labeled with dopamine antibody (DA⁺). **D:** In a cross section of the brain, a cell (arrow) in the periventricular nucleus of the posterior tuberculum is double-labeled by TH1 immunohistochemistry and retrograde tracing from a mid-thoracic level of the spinal cord with RDA. **E:** No mRNA expression for TH2 is detectable in spinal cross sections (upper panel), but in a horizontal brain section in the posterior periventricular preoptic nucleus (lower

panel). Scale bars: A = 40 μm for low and 5 μm for high magnification; B = 5 μm ; D = 20 μm ; E = 20 μm (upper) and 15 μm (lower).

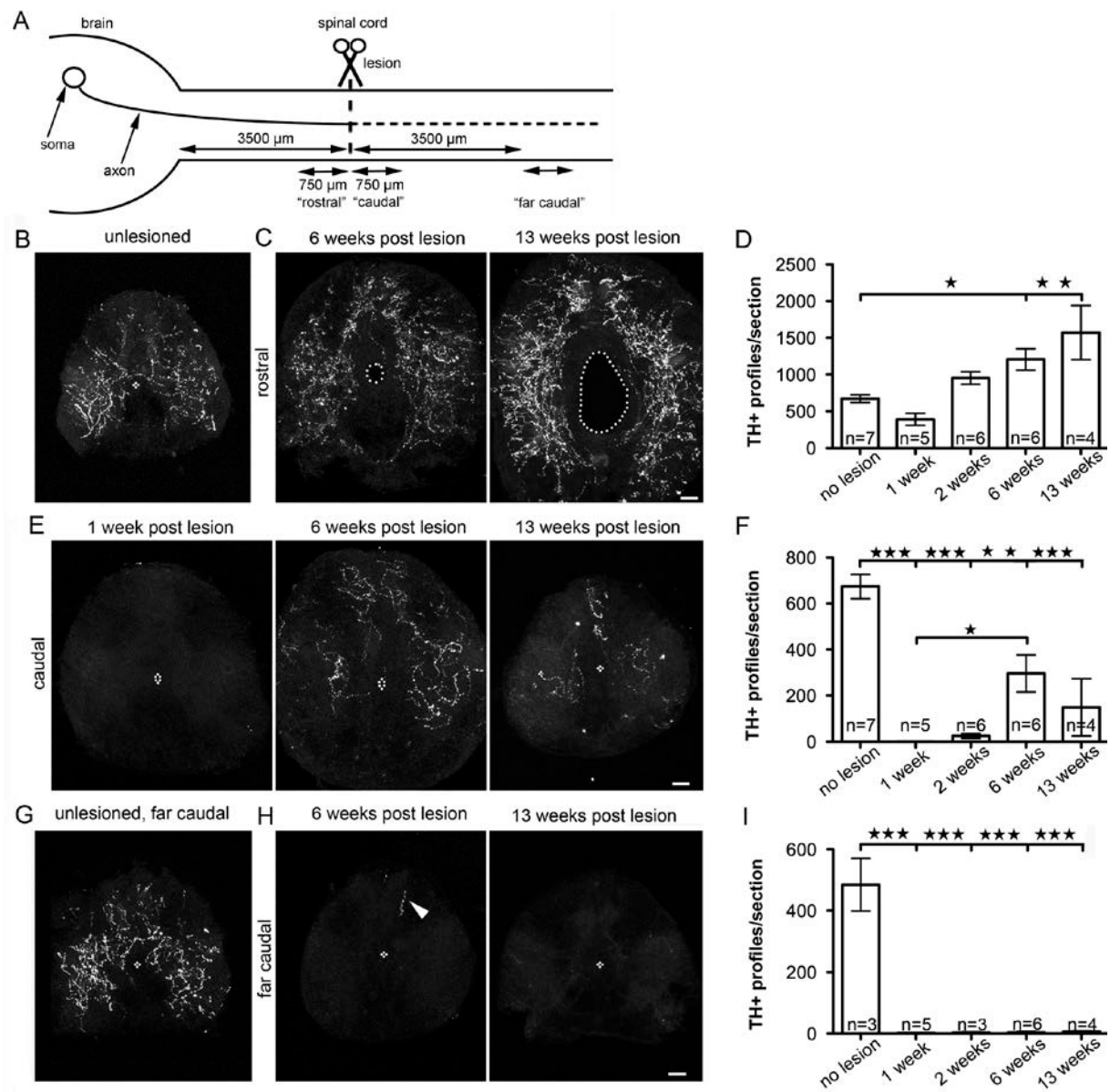


Fig. 2 Plasticity of TH1⁺ axons after spinal lesion. Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. Levels of sectioning are depicted in A. **B**: TH1⁺ axons, but no cells are labeled in the unlesioned spinal cord. This section serves as control for B and D. **C,D**: Numbers of TH1⁺ profiles increase continuously rostral to the lesion site for at least 13 weeks post-lesion. **E,F**: Caudal to the lesion site all axons are lost and axon regrowth is observed at 6 weeks post-lesion. **G-I**: Axon regrowth to a level 3.5 mm caudal to the lesion site is rarely (arrowhead) observed. *P < 0.05, **P < 0.01, ***P < 0.001; Scale bars: 25 μ m.

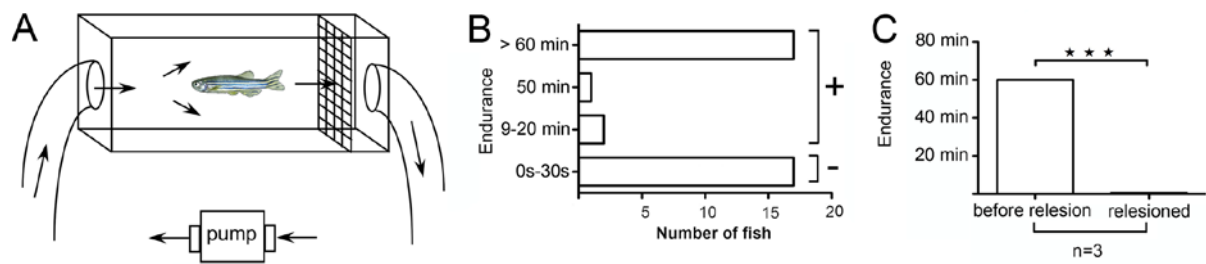


Fig. 3 Recovery of swimming capability is variable and is abolished by re-transection of the spinal cord. **A:** A schematic representation of the flow-through tank used to test swim performance is shown. **B:** Fish that were unable to hold their position against a constant water flow for more than 30 seconds were classified as not recovered. **C:** Recovered fish lose their regained swimming ability after relesion.

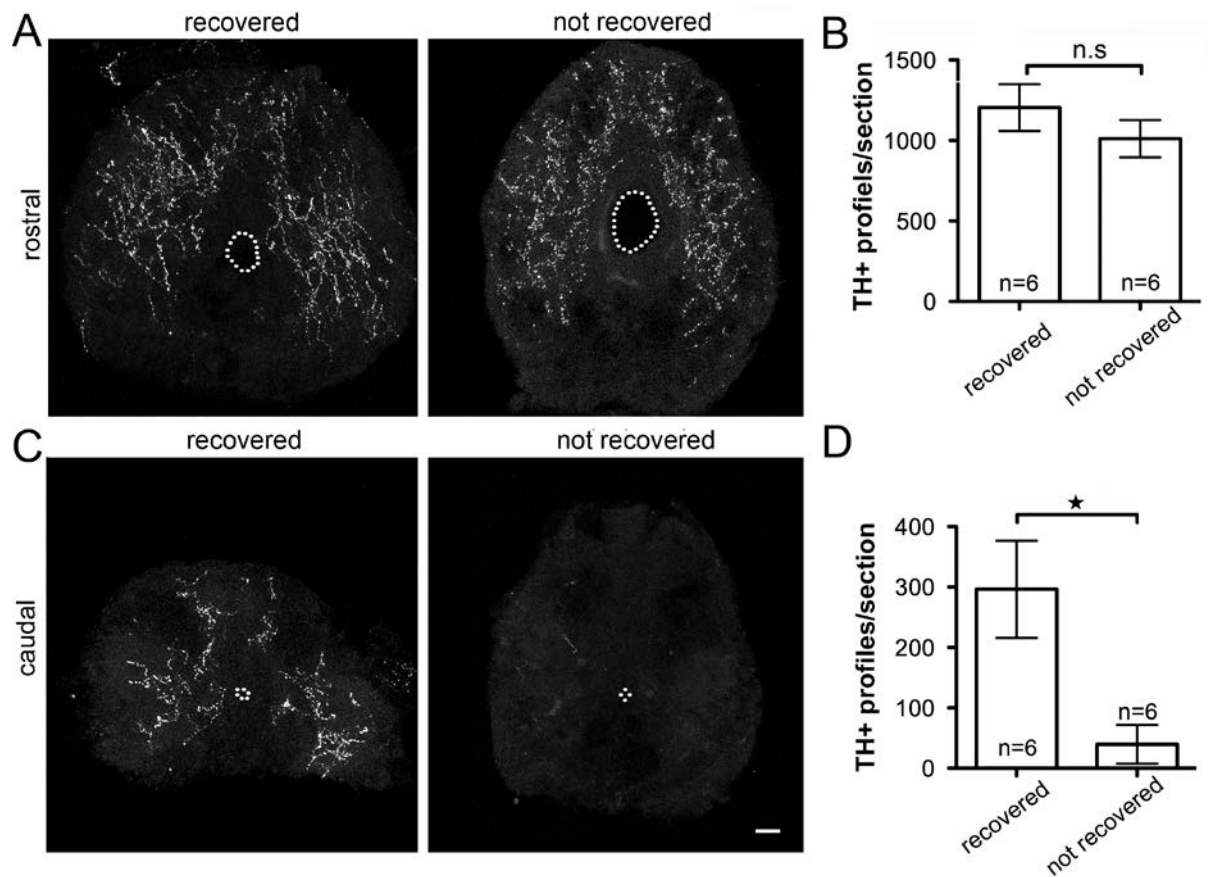


Fig. 4 Recovery of swimming capability correlates with regrowth of TH1⁺ axons beyond the lesion site. Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. Levels of sectioning are depicted in Fig. 2. **A,B:** Rostral to the lesion site numbers of TH1⁺ profiles did not differ between recovered and non-recovered fish. **C,D:** Significantly fewer TH1⁺ axons regenerated beyond the spinal transection site in non-recovered fish than in those showing recovery of swimming capability (*P < 0.05). Scale bar = 25 μ m.

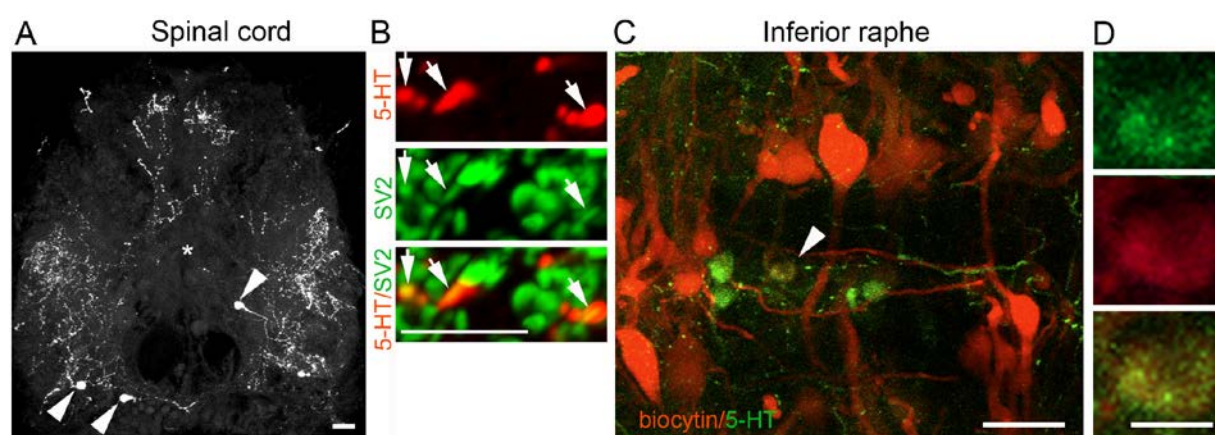


Fig. 5 Spinal 5-HT⁺ axons originate in the spinal cord and brainstem. **A,B:** 5-HT immunohistochemistry on spinal cross sections (dorsal is up) reveals labeled somata (arrowheads) in the ventral spinal cord (A). 5-HT⁺ protrusions double-label with the synaptic marker SV2 (B, arrows). The central canal is indicated by dots. **C,D:** A horizontal section through the brainstem is shown; rostral is right. Retrograde tracing from the spinal cord reveals 5-HT immunoreactive somata of neurons in the inferior raphe that project to the spinal cord. A double-labeled cell (arrowhead in C) is shown in higher magnification in D. Scale bars: A = 15 μ m; B = 5 μ m; C = 20 μ m; D = 5 μ m.

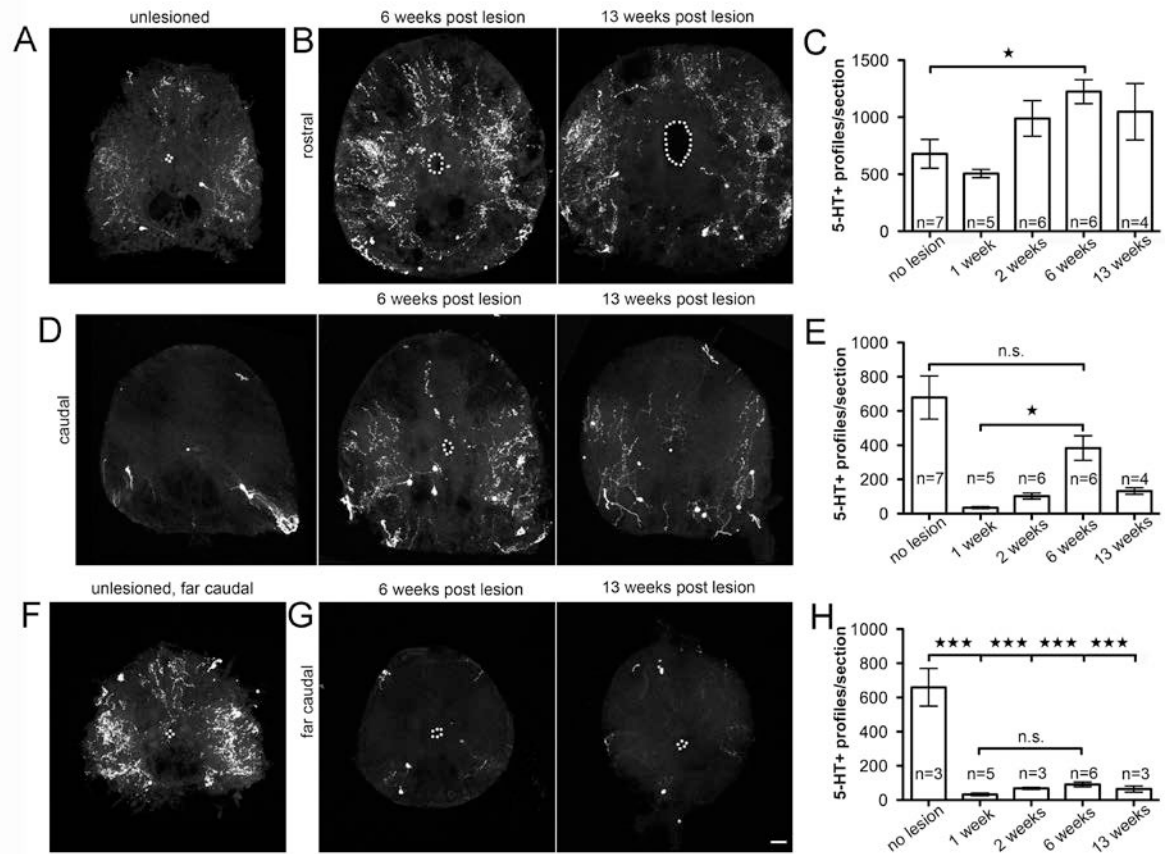


Fig. 6 Plasticity of 5-HT⁺ axons after spinal lesion. Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. Levels of sectioning are depicted in Fig. 2. **A:** 5-HT⁺ axons and cells are labeled in the unlesioned spinal cord. This section serves as control for B and D. **B,C:** Numbers of 5-HT⁺ profiles increase continuously rostral to the lesion site and plateau at 6 weeks post-lesion. **D,E:** Caudal to the lesion site most axons are lost and significant axon regrowth is observed at 6 weeks post-lesion. **F-H:** Numbers of axonal profiles remain low at a level 3.5 mm caudal to the lesion site for at least 13 weeks post-lesion. ***P < 0.001, **P < 0.01, *P < 0.05; Scale bar = 25 μ m.

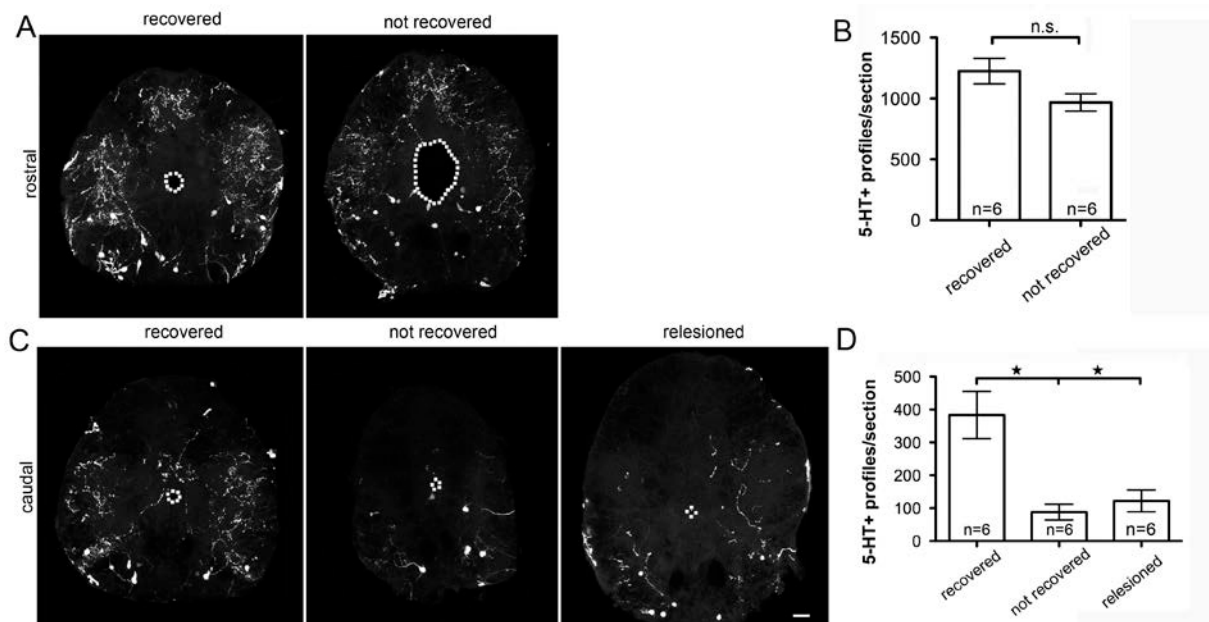


Fig. 7 Recovery of swimming capability correlates with regrowth of descending 5-HT⁺ axons beyond the lesion site. Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. Axial levels of sectioning are depicted in Fig. 2. **A,B:** Rostral to the lesion site numbers of 5-HT⁺ profiles do not differ between recovered and non-recovered fish. **C,D:** Significantly fewer 5-HT⁺ axons are present caudal to the spinal transection site in non-recovered fish than in those showing recovery of swimming capability (* $P < 0.05$ xx in the text it says 0.01?). Re-lesion of recovered fish strongly reduces the number of axons caudal to the lesion site, indicating that axonal profiles are mainly derived from descending axons. Scale bar = 25 μ m.

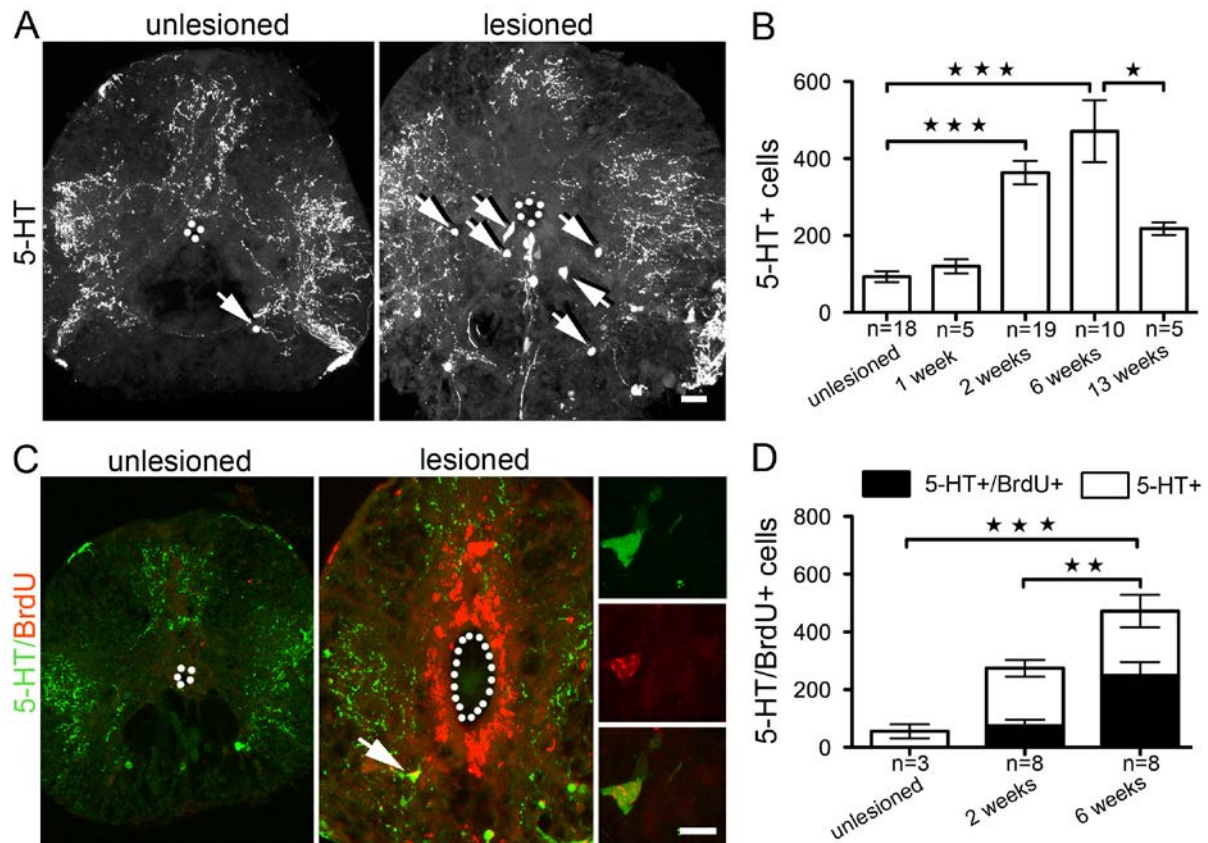


Fig. 8 5-HT⁺ cells are newly generated in the vicinity of a spinal lesion. Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. **A**: Arrows depict 5-HT⁺ neurons in the unlesioned and 6 weeks post-lesion spinal cord. **B**: Numbers of 5-HT⁺ neurons rise until 6 weeks post-lesion and are reduced again at 13 weeks post-lesion. **C**: BrdU-labeled 5-HT⁺ cells are detectable at 2 weeks post-lesion (arrow; higher magnification is show in small panels on the right). **D**: A sizable proportion of 5-HT⁺ cells are double labeled with BrdU at different post-lesion times. Scale bar in A = 20 μ m for A and low magnification in B; scale bar in C = 10 μ m for high magnifications only.

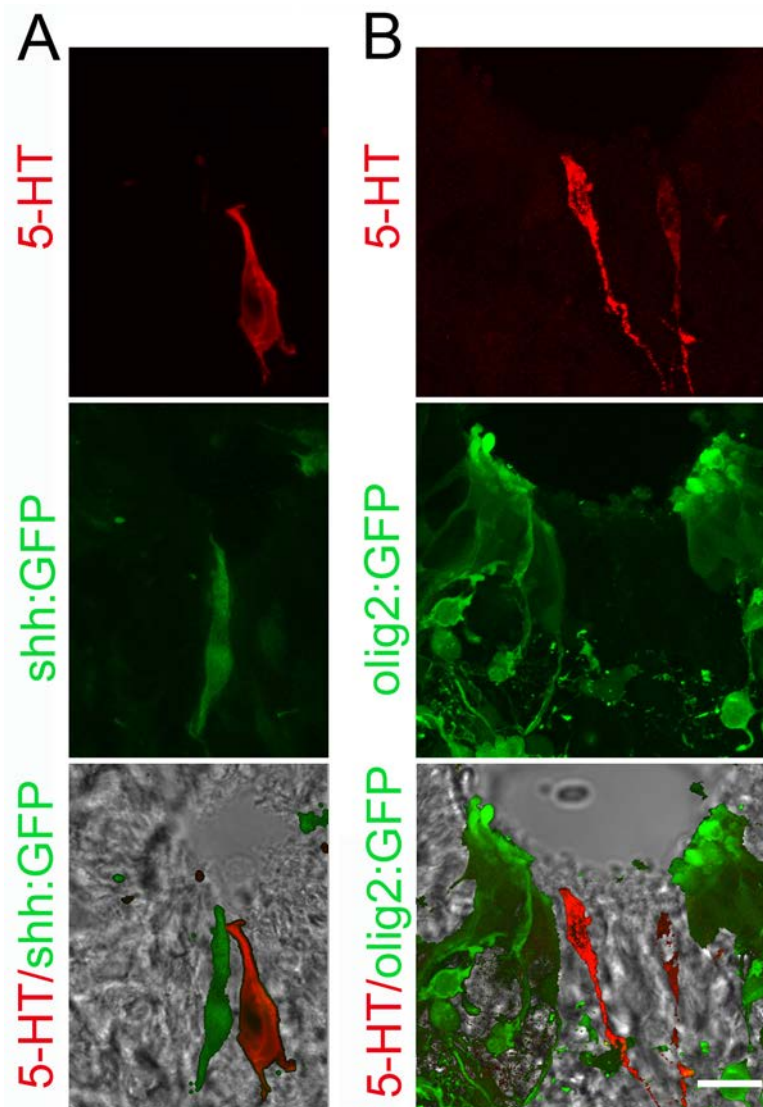


Fig. 9 5-HT⁺ cells that contact the central canal in a ventral position are found in the lesioned spinal cord. Cross sections of the ventral ventricular zone at 3 months post-lesion are shown; dorsal is up. Ventricular position is depicted in phase-contrast images underlying double-labeling in bottom row. **A:** Central canal-contacting 5-HT⁺ cells are located close to shh:GFP⁺ endpendymo-radial glial cells. **B:** Central canal-contacting 5-HT⁺ cells are located ventral to ventro-lateral zones of olig2:GFP⁺ endpendymo-radial glial cells. Scale bar = 10 μm.

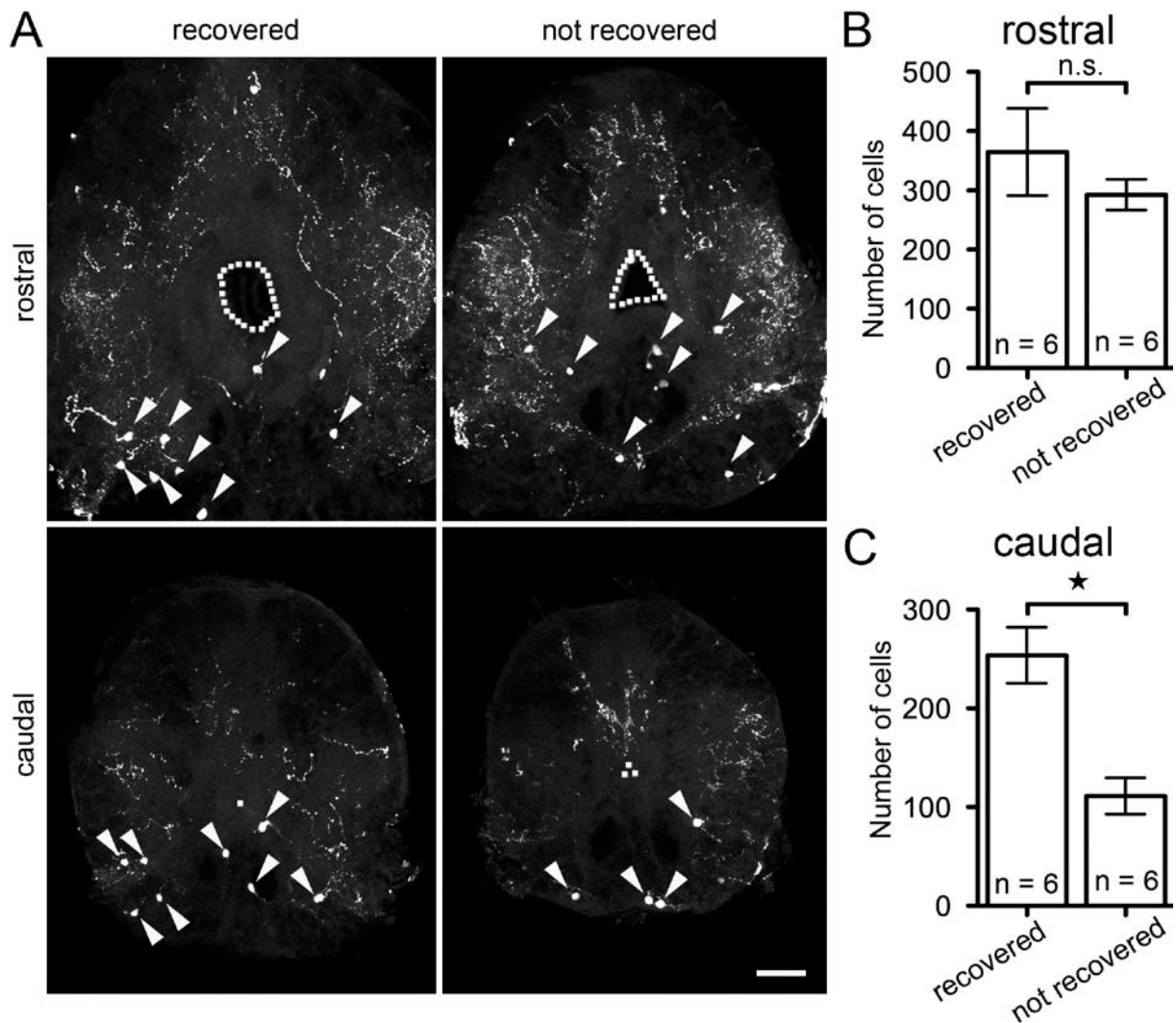


Fig. 10 Numbers of 5-HT⁺ cells caudal to the lesion site correlate with recovery of swimming function at 6 weeks post-lesion. **A**: Complete spinal cross sections are shown; rostral is up; central canal is outlined by dotted line. Axial levels of sectioning are depicted in Fig. 2. Arrowheads indicate 5-HT⁺ cells. **B,C**: Cell numbers caudal, but not rostral to the transection site are significantly less in non-recovered fish (*P < 0.05). Scale bar = 50 μ m.

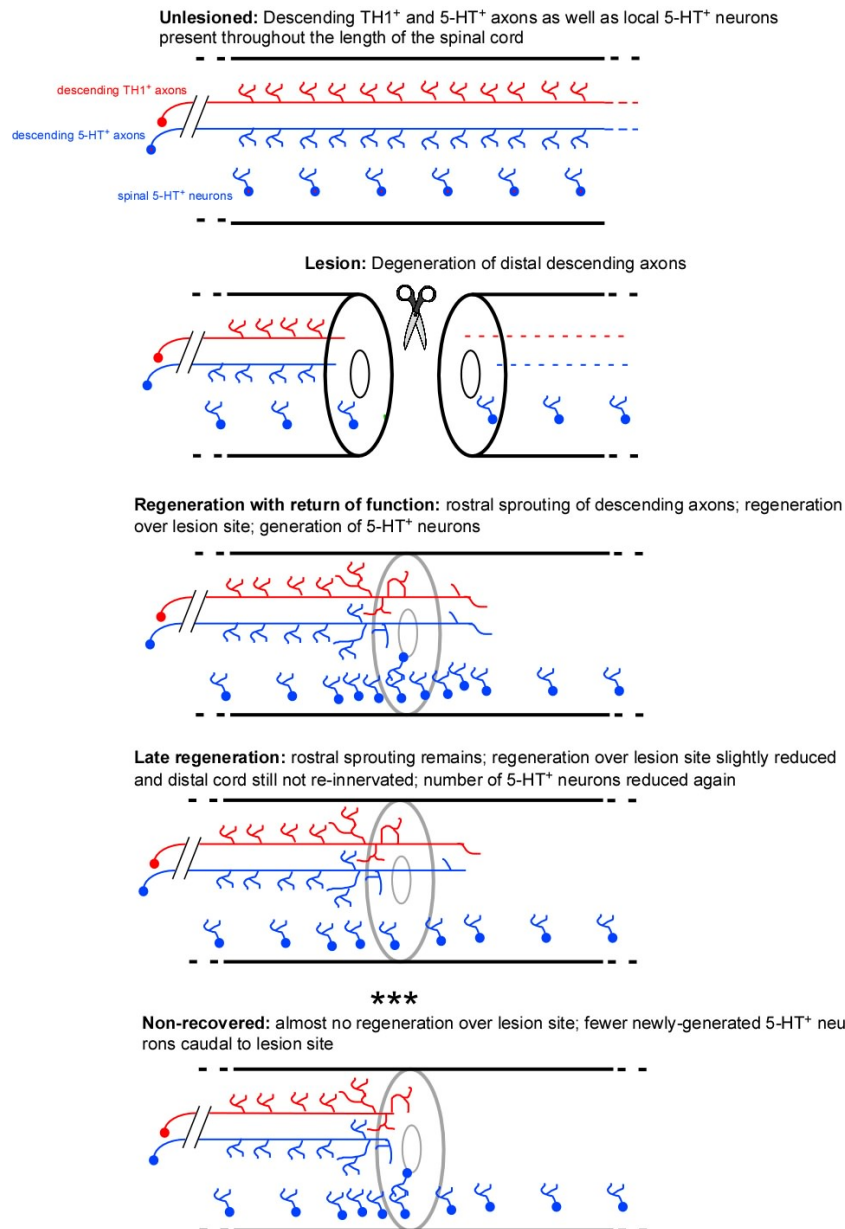


Fig. 11 Summary of quantitative changes in TH1⁺ and 5-HT⁺ systems after a spinal lesion.

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